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DOCKET : VLADIMIR BAKHUTASHVILI, AMNIOTIC APOPTOSIS MODULATING SUBSTANCES, U.S. SERIAL NO. 10/795, 819, FILED MARCH 8, 2004, CONTINUATION OF U.S. SERIAL NO. 09/928,178, FILED AUGUST 9, 2001, WHICH CLAIMS PRIORITY OF U.S. SERIAL NO. 60/224,112, FILED AUGUST 9, 2000 – DKT. #627-B-US

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COMMUNICATION TO SUBMIT SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT for Vladimir BAKHUTASHVILI, U.S. Serial 10/795,819, March 8, 2004, continuation of U.S. Serial No. 09/928,178, filed August 9, 2001, which claims priority to U.S. Provisional Application, U.S. Serial No. 60/224,112, filed August 9, 2000, for **AMNIOTIC APOPTOSIS MODULATING SUBSTANCES**, including **Exhibit A, and Exhibits 1-2.**

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Vladimir BAKHUTASHVILI

U.S. Serial No. : 10/795,819 Examiner: Ruth A. Davis

Filed : March 8, 2004 Art Unit: 1651

For : AMNIOTIC APOPTOSIS MODULATING SUBSTANCES

Law Offices of Albert Wai-Kit Chan, LLC
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April 25, 2005

Commissioner for Patents
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Respectfully submitted,

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Dkt. #627-B-US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Vladimir BAKHUTASHVILI

U.S. Serial No.: 10/795,819 Examiner: Ruth A. Davis

Filed : March 8, 2004 Art Unit: 1651

FOR : AMNIOTIC APOPTOSIS MODULATING SUBSTANCES

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Whitestone, New York 11357

April 25, 2005

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Sir/Madam:

COMMUNICATION TO SUBMIT SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT

In accordance with his duty of disclosure under 37 C.F.R. §1.56, Applicant would like to direct the Examiner's attention to the following references which are listed below and on Form PTO/SB/08B (**Exhibit A**), with each individual reference further attached as **Exhibit 1** and **Exhibit 2.**

1. Supplemental European Search Report for LAJOR BIO TECH, INC., European Application No. 01959894.5, Filed March 7, 2003, Dated March 14, 2005. **[Exhibit 1]**

2. RUNIC, et al., "Apoptosis and Fas Expression in Human Fetal Membranes"; Journal of Clinical Endocrinology and Metabolism, Vol. 83, no. 2, February 1998 (1998-02) pages 660-666, XP002319582. **[Exhibit 2]**

Applicant : Vladimir BAKHUTASHVILI
U.S. Serial No. : 10/795,819
Filed : March 8, 2004
Page : 2

3. IWAMA, et al., "Serum Concentrations of Fas Antigen and Soluable Fas Ligand in Mother and Newborn"; Archives of Gynecology and Obstetrics, Vol. 263, no. 3, February 2000 (2000-02), pages 108-110, XP002319583. **[Exhibit 3]**

If a telephone interview would be of assistance in advancing prosecution of the subject application, Applicant's undersigned attorney invites the Examiner to telephone him at the number provided below.

No fee is deemed necessary in connection with the filing of this Supplemental Information Disclosure Statement. However, if any additional fee is required, authorization is hereby given to charge the amount of any such fee to Deposit Account No. 50-1891.

Respectfully submitted,

Albert Wai-Kit Chan

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Exhibit A

PTO/SB/08B (04-03)
Approved for use through 04/30/2003. OMB 0651-0031
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Substitute for form 1449/PTO

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 1 of 1

Complete If Known	
Application Number	10/795,819
Filing Date	March 8, 2004
First Named Inventor	BAKHUTASHVILI, Vladimir
Art Unit	1651
Examiner Name	Ruth A. Davis
Attorney Docket Number	627-B-US

NON PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
	1	Supplemental European Search Report for LAJOR BIO TECH, INC., European Application No. 01959894.5, Filed March 7, 2003, Dated March 14, 2005. [Exhibit 1]	
	2	RUNIC, et al., "Apoptosis and Fas Expression in Human Fetal Membranes"; Journal of Clinical Endocrinology and Metabolism, Vol. 83, no. 2, February 1998 (1998-02) pages 660-666, XP002319582. [Exhibit 2]	
	3	IWAMA, et al., "Serum Concentrations of Fas Antigen and Soluable Fas Ligand in Mother and Newborn"; Archives of Gynecology and Obstetrics, Vol. 263, no. 3, February 2000 (2000-02), pages 108-110, XP002319583. [Exhibit 3]	

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

1 Applicant's unique citation designation number (optional). 2 Applicant is to place a check mark here if English language Translation is attached.

Exhibit 1

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Datum/Date
14.03.05

Zeichen/Ref./Réf.	Anmeldung Nr./Application No./Demande n°./Patent Nr./Patent No./Brevet n°.
BR74891/DC1/JT/	01959894.5-2405-US0141666

Anmelder/Applicant/Demandeur/Patentinhaber/Proprietor/Titulaire
Lajor Bio Tech, INC.

COMMUNICATION

The European Patent Office herewith transmits as an enclosure the European search report for the above-mentioned European patent application.

If applicable, copies of the documents cited in the European search report are attached.

☐ Additional set(s) of copies of the documents cited in the European search report is (are) enclosed as well.

REFUND OF THE SEARCH FEE

If applicable under Article 10 Rules relating to fees, a separate communication from the Receiving Section on the refund of the search fee will be sent later.

European Patent Office

SUPPLEMENTARY EUROPEAN SEARCH REPORT

Application Number
EP 01 95 9894

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
X	RUNIC RADMILA ET AL: "Apoptosis and Fas expression in human fetal membranes" JOURNAL OF CLINICAL ENDOCRINOLOGY AND METABOLISM, vol. 83, no. 2, February 1998 (1998-02), pages 660-666, XP002319582 ISSN: 0021-972X * the whole document *	1	C07K14/00 A61K38/00 A61K49/00
X	IWAMA H ET AL: "Serum concentrations of soluble Fas antigen and soluble Fas ligand in mother and newborn" ARCHIVES OF GYNECOLOGY AND OBSTETRICS, vol. 263, no. 3, February 2000 (2000-02), pages 108-110, XP002319583 ISSN: 0932-0067 * the whole document *	1	

TECHNICAL FIELDS SEARCHED (Int.Cl.7)

A61K

The supplementary search report has been based on the last set of claims valid and available at the start of the search.

Place of search	Date of completion of the search	Examiner
The Hague	4 March 2005	Rempp, G

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

Exhibit 2

0021-972X/98/$03.00/0
Journal of Clinical Endocrinology and Metabolism
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Vol. 83, No. 2
Printed in U.S.A.

Apoptosis and Fas Expression in Human Fetal Membranes*

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ABSTRACT

Apoptosis (*i.e.* programmed cell death) plays a key role in maintaining reproductive function in the ovary, mammary and prostate glands, uterus, and testis. The purpose of the present report was to determine, based on biochemical and morphological parameters, whether cells in human fetal membranes undergo apoptosis and express Fas (CD95), a cell surface receptor that mediates apoptosis. Using the terminal deoxynucleotidyl transferase deoxy-UTP-nick end labeling immunohistochemical technique, apoptotic nuclei were identified in amnion epithelial, chorionic trophoblast, and decidua parietalis cell layers of human fetal membranes at term. Electron microscopy of fetal membranes revealed ultrastructural characteristics in amnion epithelium and chorion trophoblast cell layers consistent with apoptosis, including condensation of chromatin along the periphery of the nucleus and nuclear shrinkage. The apoptotic index (percentage of terminal deoxynucleotidyl transferase deoxy-UTP-nick end labeling-positive nuclei of the total nuclei) ranged from 8–29% in amnion epithelial, chorionic trophoblast, and decidual cell layers from women at 23–30, 31–36, and 37–42 weeks gestation. The apoptotic index was statistically greater in the 37–42 week group than in the 23–30 week group in chorionic trophoblast ($P < 0.05$) and decidual cell ($P < 0.01$) layers. In contrast, the apoptotic index in the amnion epithelial cell layer was statistically greater ($P < 0.05$) in the 23–30 week group than in the 31–36 week group, suggesting that apoptosis may be independently regulated in amnion epithelial, chorionic trophoblast, and decidual cell types. Based on the importance of Fas in mediating apoptosis, we investigated whether Fas was expressed by human fetal membrane cells. Immunohistochemical staining of fetal membranes with anti-Fas antibody localized Fas in amnion epithelial, chorionic trophoblast, and decidua parietalis cell layers. A 256-bp band corresponding to the cytoplasmic domain of Fas was detected in samples of amnion, chorion, decidua, and placenta by RT-PCR. Northern blotting revealed a molecular weight of approximately 1.9 kilobases for Fas messenger ribonucleic acid in amniotic tissue. These data suggest that apoptosis and Fas signaling may play a role in remodeling of fetal membrane architecture across gestation. (*J Clin Endocrinol Metab* 83: 660–666, 1998)

THE DYNAMIC nature of the fetal membranes enables accommodation to the changing needs of the fetus across gestation. Accordingly, maintenance of fetal membrane integrity throughout pregnancy is required for normal fetal development (1, 2). Conversely, fetal membrane rupture is associated with parturition whether occurring before or at term (1, 2). Although the etiology of fetal membrane rupture remains unelucidated, it is clear that gross morphological, biochemical, and structural changes take place in human fetal membranes across gestation and accompany their rupture (3, 4). These include a dramatic thinning and reduction in tensile strength and a marked reduction in the chorionic intermediate trophoblast and decidua parietalis cell layers (3). Apoptosis is characterized by cellular events including nuclear condensation and fragmentation and cell shrinkage in isolated cells within a tissue (5, 6). Apoptosis occurs without activation of the immune system or generalized inflammation (5, 6). This is in marked contrast to necrosis, in which cell swelling and spillage of cytoplasmic contents into neighboring cells elicits an inflammatory response (5, 6).

Fas, a 45-kDa cell surface receptor of the tumor necrosis factor (TNF)/nerve growth factor family, mediates apoptosis of target cells after binding of Fas ligand (FasL) (7). Although Fas/FasL function was originally described in the context of lymphocyte-mediated apoptosis of lymphocytes, recent data indicated that the Fas/FasL signaling system may promote apoptosis of epithelial cells in ovarian follicles (8) and the thyroid gland (9). Local expression of FasL in cells of the testis (10) and the anterior region of the eye (11) was suggested in part to confer immune tolerance by promoting apoptosis of activated Fas-bearing lymphocytes that infiltrate these sites. Our previous results indicated that FasL was expressed in the human placenta and fetal membranes across gestation (12).

The purpose of the present study was to determine, based on biochemical and morphological parameters, whether cells in human fetal membranes undergo apoptosis and express Fas. Based on immunohistochemical, ultrastructural, and biochemical data, we report that apoptosis is a physiological process in human fetal membranes in the third trimester of pregnancy. In addition, our documentation of Fas expression in chorion, amnion, and decidua of fetal membranes at term may suggest a role for Fas/FasL signaling in apoptosis and remodeling of fetal membrane architecture across gestation.

Materials and Methods

Procurement of tissues

Fetal membranes and placentas were obtained from 17 preterm (<37 weeks) and 21 term placentas (≥37 weeks). Samples at term were ob-

Received July 14, 1997. Revision received October 28, 1997. Accepted November 7, 1997.

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* This work was supported in part by NIH Grant HD-33909 (to S.G.) and by the Kaplan Cancer Center (NCI grant P30 CA 16087).

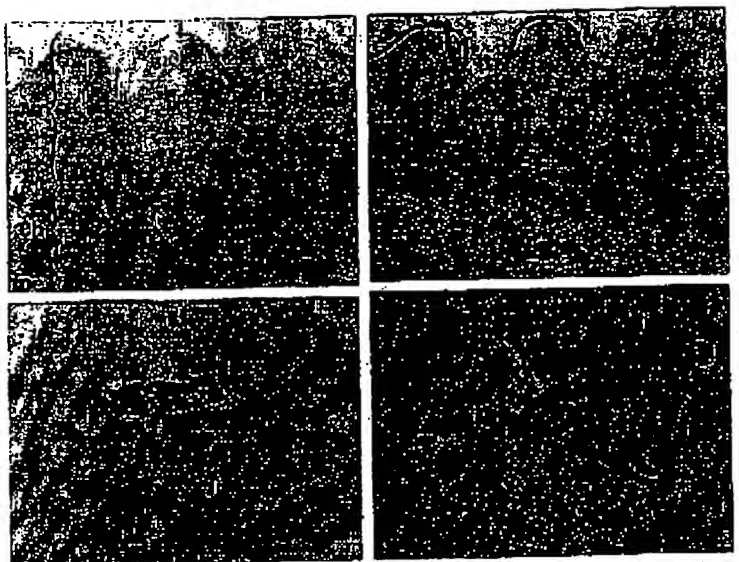

FIG. 1. Immunohistochemical staining of amnion epithelial cells and chorionic trophoblasts in term fetal membranes by TUNEL. Immunohistochemical staining of a fetal membrane roll by TUNEL is shown in a, c, and d. A consecutive section was stained with hematoxylin-eosin (b). The *upper arrow* in a denotes the presence of a condensed TUNEL-positive amnion epithelial cell. The *lower arrow* denotes TUNEL staining beneath the amadotic basal lamina, suggesting the presence of apoptotic bodies in macrophages at this site. TUNEL-positive chorionic trophoblasts are shown in d. Magnification: a and b, ×200; c and d, ×1150. Am, Amnion; Ch, chorion; De, decidua.

trained from women undergoing uncomplicated spontaneous vaginal delivery or cesarean section with or without labor. Preterm samples were obtained from pregnancies complicated by preeclampsia, diabetes, chorioamnionitis, and premature rupture of membranes. All specimens were obtained with the consent of the surgeon and the pathologist according to internal review board protocols at New York University Medical Center.

Terminal deoxynucleotidyl transferase deoxy-UTP-nick end labeling (TUNEL) and immunohistochemistry

Fetal membrane tissue obtained within 1 h after delivery was dissected from the placental disc at the periplacental edge and cut into strips 1–2 cm wide including the area from the periplacental edge to the rupture site. Tissues were washed in saline, rolled, fixed in 10% formalin, and embedded in paraffin as previously described (13). Embedded tissue sections (5 μm) were applied to poly-L-lysine-treated glass slides (Newcomer Supply, Middleton, WI). Deparaffinization of tissue sections was performed for 2 h at 58 C before dehydration with xylene and rehydration with ethanol. Alternatively, membrane rolls were flash-frozen in OCT (Baxter Scientific Products, Edison, NJ) in dry ice/2-methylbutane (Sigma Chemical Co., St. Louis, MO). Both methods of sample preparation yielded similar patterns of TUNEL and Fas staining.

TUNEL of fetal membranes was performed using the ApopTag kit from Oncor (Gaithersburg, MD). Tissue sections were treated with 20 μg/mL proteinase K for 25 min at room temperature and washed with distilled water, and endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide in 100% methanol for 5 min. Slides were then rinsed with phosphate-buffered saline (PBS) and incubated for 1 h at 37 C in buffer containing digoxigenin-labeled deoxy-UTP and terminal deoxynucleotidyl transferase. Samples were then washed three times with PBS and incubated for 30 min at room temperature with antidigoxigenin antibody-peroxidase conjugate. After rinsing with PBS, slides were incubated at room temperature for 5 min with diaminobenzidine. Slides were counterstained with methyl green (Sigma). Controls were carried out in which terminal deoxynucleotidyl transferase was omitted from the labeling reaction.

For Fas immunohistochemistry, 5-μm sections of preterm and term fetal membrane rolls ($n = 5$) were incubated overnight at 4 C with 2 μg/mL rabbit anti-Fas antibody (Santa Cruz Biotechnology, Santa Cruz, CA), with and without 20 μg/mL of a peptide corresponding to amino acids 21–38 of human Fas. After incubation with primary antibody, slides were incubated with anti-rabbit antibody/peroxidase conjugate at a dilution of 1:500. Color development in peroxidase reactions was carried out using diaminobenzidine supplied with the Vectastain ABC Kit (Vector Laboratories, Burlingame, CA). Samples were counterstained with hematoxylin.

Quantitation of apoptotic index and statistical analysis

For each TUNEL and methyl green-stained tissue section, brown apoptotic nuclei and blue-green healthy nuclei were blindly counted by 2 individuals (R.R. and E.D.) in each of 12 independent microscopic fields for amnion epithelial, chorion trophoblast, and decidua parietalis cell layers using a ×40 objective. Between 300–1000 nuclei were counted for each sample. Eighty-one different specimens were employed to calculate interobserver correlation, and intraobserver correlation was carried out in 5 independent specimens. Interobserver ($r = 0.884$) and intraobserver ($r = 0.984$; $r = 0.950$) correlations were high. The apoptotic index (number of apoptotic nuclei per total nuclei × 100) was expressed

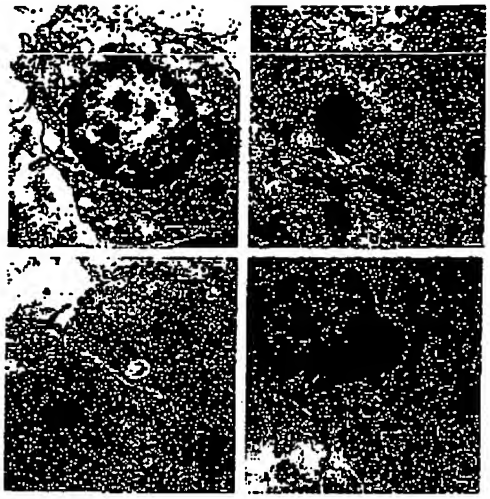

FIG. 2. Ultrastructural characteristics of nuclei of human fetal membranes at term. Electron micrographs of ultrathin sections of chorionic trophoblast (a and b) and amnion epithelial cells (c and d) obtained from a spontaneous vaginal delivery at term are shown. Note the marked condensation of nuclear chromatin in apoptotic nuclei (denoted by *arrows*) compared to the dispersed chromatin in normal nuclei (N). Original magnifications: a and d, ×20,000; b, ×15,750; c, ×12,500.

as the mean ± SD. Statistical analysis was performed using ANOVA to statistically compare the apoptotic indexes of amnion, chorion, and decidua samples, and linear regression analysis was performed to compare the inter- and intraobserver correlations (SigmaStat software, Jandel, San Rafael, CA).

Electron microscopy

Fragments of fetal membranes were fixed in 2.5% glutaraldehyde in sodium cacodylate buffer, pH 7.2. After overnight fixation by immersion, tissues were postfixed in 1% osmium tetroxide, dehydrated through ascending grades of alcohol, embedded in epon, and sectioned on an ultramicrotome. Thin sections (~60 nm) were stained with saturated uranyl acetate and aqueous lead citrate for electron microscopy. A Zeiss 10A transmission electron microscope (Zeiss, New York, NY) was used to view thin sections and for photography (14).

RT-PCR and Northern blotting for Fas

For RT-PCR analysis of Fas expression, placental and fetal membrane tissue were rinsed with saline, frozen in liquid nitrogen, and stored at −80 C. Frozen tissues were homogenized by Polytron disruption (Brinkmann Instruments, Westbury, NY), and total ribonucleic acid (RNA) was isolated using UltraSpec RNA (Biotecx, Houston, TX). Samples were then extracted with a mixture of phenol-chloroform-isoamyl alcohol (25:24:1) and then with chloroform alone. One microgram of total RNA was primed with 2.5 μmol/L random hexamers and reverse transcribed with 2.5 U/L murine leukemia virus reverse transcriptase (Perkin-Elmer/Cetus, Branchburg, NJ) in a 20-μL reaction mix according to the manufacturer's protocol. Twenty microliters of complementary DNA (cDNA) were then PCR amplified with 15 pmol Fas-cytoplasmic domain-specific primers (*sense*, 5'-CACTATTGCTGGAGTCATC-3'; *antisense*, 5'-CTCAGTCACTAGTAATGTCC-3') in a solution containing 200 μmol/L of each deoxy-NTP, 50 mmol/L KCl, 10 mmol/L Tris (pH 8.8), 2.5 U Taq polymerase (buffer A), and 2 mmol/L $MgCl_2$ in a total volume of 100 μL. Primers were synthesized by Genosys (The Woodlands, TX) as previously described (15), and amplification was carried out in a GeneAmp PCR System 9600 (Perkin-Elmer/Cetus). First strand cDNA was denatured at 95 C for 1 min and 45 s. In each subsequent cycle of amplification, DNA was denatured at 95 C for 15 s, annealed at 58 C for 20 s, and polymerized at 72 C for 15 s. After 40 cycles of amplification, polymerization was carried out at 72 C for 7 min, and samples were immediately placed at 4 C.

For a positive control, 1 μg total RNA was also reverse transcribed as described above, and 20 μL cDNA were PCR amplified in buffer A supplemented with 1.5 mmol/L $MgCl_2$ containing 15 pmol glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific primers (*sense*, 5'-GGTCGGAGTCA-ACGGATTTGGTCG-3'; *antisense*, 5'-CCTCGCA-CGCCTGCTTCACCAC-3'; Genosys) as previously described (16). For each cycle of amplification, DNA was denatured at 95 C for 30 s, annealed at 60 C for 30 s, and polymerized at 72 C for 30 s. After 35 cycles of amplification, polymerization was performed at 72 C for 7 min, and samples were immediately placed at 4 C. After amplification, Fas and GAPDH RT-PCR products were visualized by electrophoresis on a 2% agarose gel containing 0.5 μg/mL ethidium bromide.

For Northern blot analysis, 1–4 μg polyadenylated RNA were isolated from approximately 400 mg fresh amnion tissue using the Micro-Fast Track kit (Invitrogen, San Diego, CA). Formaldehyde gel electrophoresis was carried out as previously described (17), and RNA was transferred to a ZetaProbe nylon membrane (Bio-Rad, Richmond, CA). Blots were prehybridized for 2 h at 42 C in buffer containing Denhardt's solution and 5SC (standard saline citrate) as previously described (18). Hybridization was then carried out overnight at 42 C in the same buffer containing 10^6 cpm/mL of a ^{32}P-labeled 266-bp PCR product corresponding to the cytoplasmic domain of Fas. Levels of GAPDH messenger RNA (mRNA) were analyzed as previously described (19). Scanning and printing of PCR and Northern blot data were carried out using Sigma Scan/Sigma Image software.

Results

Apoptosis in human fetal membranes revealed by TUNEL and electron microscopy

Human fetal membranes consist of amnion, chorion, and decidua parietalis tissue layers (denoted Am, Ch, and De, respectively, in Fig. 1a).

Nuclear fragmentation was examined in fetal membranes using the TUNEL/immunohistochemical method. Fragmented nuclei, identified by the appearance of a brown peroxidatic product, were observed in scattered areas in amnion epithelial cells, intermediate trophoblasts of the chorion, and decidua parietalis cells in fetal membrane rolls obtained at 40 weeks gestation (Fig. 1, a, c, and d). Healthy (*i.e.* nonfragmented) nuclei retained the blue-green color of the methyl green counterstain. Under high magnification, amnion epithelial cells (Fig. 1c, *upper arrow*) and chorionic trophoblasts (Fig. 1d) were visualized by TUNEL staining. In addition, scattered staining was observed in the area beneath the basal lamina of the amnion (Fig. 1c, *lower arrow*), possibly reflecting the presence of apoptotic bodies within macrophages at this site.

Electron micrographs of human fetal membranes at term revealed ultrastructural changes in chorionic trophoblasts (Fig. 2, a and b) and amnion epithelial cell (Fig. 2d) layers consistent with apoptosis, including condensation of nuclear chromatin along the periphery of the nucleus and shrinkage of cellular cytoplasm. Peripheral condensation of chromatin, a hallmark of apoptosis (20), was apparent within highly condensed nuclei (Fig. 2). Conversely, chromatin remained

Morphological and biochemical data obtained in the current report are consistent with the conclusion that cells of the amnion epithelial, chorionic trophoblast, and decidua parietalis layers in human fetal membranes undergo apoptosis. Immunohistochemical data indicated that between 8–29% of these cell types were TUNEL positive. The observed dispersed pattern of TUNEL staining in human fetal membranes was similar to that noted in other tissues undergoing apoptosis (26, 27). Data obtained by electron microscopy in the present study are consistent with changes noted during apoptosis, including condensation of chromatin along the periphery of the nucleus and, ultimately, shrinkage of the nucleus and the cell itself. Although TUNEL staining is often correlated with apoptosis, it has been suggested that this method also detects fragmented DNA in necrotic cells within a tissue (28). In the present study, the ultrastructural changes described above and the dispersed pattern of TUNEL staining are consistent with apoptosis and not necrosis, in which gross swelling of cells and their organelles occurs in patches within a tissue (5, 6). In light of these results, it is somewhat surprising that extracts of human amnion at term did not reveal an apoptotic DNA ladder pattern after ethidium bromide staining of agarose gels (not shown). This may indicate that internucleosomal cleavage of DNA does not accompany apoptosis in human fetal membranes or simply reflects an inherent difficulty in detecting DNA ladders in samples containing low levels of DNA fragmentation (29). Alternatively, it is well documented that internucleosomal cleavage of DNA to small fragments containing multiples of 180–200 bp does not always occur in cells undergoing apoptosis (30, 31).

Our results also demonstrated that the apoptotic index of chorionic trophoblasts and decidua parietalis cells was higher in term tissue compared to specimens obtained from the preterm (23–30 week) group. In contrast, the apoptotic index of amnion epithelial cells was highest in the 23–30 week group, suggesting that apoptosis in amnion, chorion, and decidua may be differentially regulated. It is of note in the present study that although the use of fetal membranes from pregnancies complicated by infection, ischemia, preeclampsia, diabetes, and premature rupture of membranes may be criticized, it is impossible to obtain preterm fetal membranes from uncomplicated human pregnancies. That these pathological conditions did not induce apoptosis in chorion and decidua is suggested by the similarity in both distribution and quantity of apoptotic cells across each condition. Conversely, high levels of apoptosis in amnion epithelial cells in the 23–30 week group may reflect pathology before term. Therefore, we suggest that apoptosis occurs as part of a program of senescence in chorionic and decidual cells that is not triggered in association with labor. Furthermore, the etiology of amnion apoptosis appears to be more complicated and may be associated with preterm pathology. However, due to the relatively small number of specimens in each group, further studies need to be conducted to associate a particular pathology with apoptosis in the amniotic epithelium and to characterize the progressive nature of apoptosis in the chorion and decidua.

TUNEL and ultrastructural results from other laboratories suggested that first trimester syncytiotrophoblasts in first trimester human placental villi undergo extensive apoptosis, whereas significantly less apoptosis was observed in term placental villi (28). TNFα and interferon-γ were demonstrated to induce apoptosis of cytotrophoblasts isolated from human term placentas (32).

A report by Paavola *et al.* documented an apoptotic program in rat amnion cells before parturition characterized by degradation of type I collagen by interstitial collagenase (33). There is precedent for apoptosis and necrosis occurring concomitantly in placental as well as cardiac tissue (28, 34). Our previous data suggested that glucocorticoids may alter the integrity of fetal membranes by reducing the synthesis of collagen III and fibronectin by amnion epithelial cells (35).

Fas (CD95), a cell surface receptor, is a member of the TNF receptor and nerve growth factor receptor family (7). It has been established that Fas and TNF receptor modulate the immune response by triggering apoptosis of lymphocytes after binding of FasL and TNF, their respective ligands (7). Recent data indicated that the apoptotic program induced by FasL and TNF required the interaction of common interleukin-converting enzyme and interleukin-converting enzyme-like proteases with receptor complexes (36, 37). It is interesting to note that although Fas-mediated apoptosis was originally described in the context of autoregulation of T lymphocyte proliferation (7), recent studies suggested that production of FasL by cells of the testis (10), the anterior region of the eye (11), the brain (38) and tumors (39, 40) may also serve an immunoprotective function by promoting apoptosis of Fas-bearing lymphocytes that infiltrate these sites. Fas-mediated apoptosis has also been implicated in the regulation of ovarian and thyroid function (8, 9).

We reported that cytotrophoblasts in human placenta and fetal membranes express FasL across gestation (12). Therefore, we hypothesized that the presence of FasL in human placenta and fetal membranes serves to protect the fetus against activated Fas-bearing maternal lymphocytes at maternal-fetal interfaces (12). Based on the involvement of Fas in mediating apoptosis (7) and our demonstration of FasL expression in human placenta and fetal membranes (12), in the present study we determined whether Fas was expressed in human fetal membranes. We documented by immunohistochemistry that Fas was present at high levels in amnion epithelial cells, chorion trophoblasts, and decidua parietalis cells of second and third trimester human fetal membranes. In addition, RT-PCR and Northern blotting techniques demonstrated the expression of Fas in term amnion, chorion, decidual, and placental tissue. These results do not provide relative levels of expression of Fas in these tissues since quantitative procedures were not used. The finding that chorionic cytotrophoblasts and amnion epithelial cells express Fas and FasL in term fetal membranes suggests that these cells may self-regulate apoptosis at this site. However, expression of Fas alone does not guarantee activation of Fas-mediated apoptosis. It is clear that other factors, including the level of expression of FasL, will determine whether the Fas-FasL apoptotic pathway is activated (7). In addition, it is of note that although human colon carcinoma and leukemia cells concomitantly express functional Fas and FasL, they do not undergo "suicide apoptosis" (39, 40).

In conclusion, our results document for the first time the expression of apoptosis and Fas in human fetal membranes.

Future studies will elucidate the role that Fas/FasL signaling may play in physiologically and pathologically triggering apoptosis in fetal membranes in association with human parturition.

Acknowledgments

The authors acknowledge Rebecca Case for technical assistance, and David Ziegler and Ronald Maddock for their help with the preparation of this manuscript.

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Exhibit 3

XP-002319583

Arch Gynecol Obstet (2000) 263:108–110

ORIGINAL ARTICLE

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Serum concentrations of soluble Fas antigen and soluble Fas ligand in mother and newborn

Received: April 1999 / Accepted: 29 June 1999

Abstract We measured soluble Fas antigen and soluble Fas ligand, which are considered to be an apoptotic substance, in maternal serum, umbilical cord serum and amniotic fluid during cesarean section at full term pregnancy. Seventeen healthy parturients with no fetal distress were studied. Soluble Fas antigen showed no different levels between these measurement sites. Soluble Fas ligand showed a difference, in which umbilical serum level was significantly higher than maternal serum and amniotic fluid levels. The present results suggest high serum levels of soluble Fas ligand in newborn. However, the reason for this evidence is entirely unknown.

Key words Fas antigen · Fas ligand · Pregnancy · Parturient · Neonate · Fetus · Cord blood

Introduction

Fas antigen (Fas) is expressed as a membrane-bound form ubiquitously on many tissues and cells [24], and Fas ligand (FasL) is expressed as a membrane-bound form mainly on activated or cytotoxic T-lymphocytes, natural killer cells and neutrophils [1, 3, 8, 12, 16]. Binding of FasL to Fas induces apoptosis in Fas-bearing cells [13, 14]. Furthermore, soluble forms of Fas (sFas) and FasL (sFasL) have been detected in serum [14], and pathophysiological roles of these substances are examined recently [4, 19, 20]. This study was preliminary designed to measure serum sFas and sFasL levels in mother and newborn at full term pregnancy.

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Patients and methods

After approval of the Institutional Committee, seventeen healthy and full term parturients with no evidence of fetal distress who provided informed consent and received cesarean section were enrolled into this study. The cause of cesarean section was repeated cesarean section, pelvic presentation or cephalopelvic disproportion.

The anesthesia for cesarean section was performed by spinal anesthesia, in which 2.0 mL of 0.3% dibucaine hyperbaric solution was injected through a 25-gauge spinal needle via the 4th-5th or 3rd-4th lumbar interspace. After confirming anesthetized spread, maternal arterial blood was collected from the femoral artery, followed by start of the surgery. Before amniotomy, amniotic fluid was aspirated through a 23-gauge needle. Immediately after delivery, umbilical venous and arterial blood were collected from the doubly clamped segment of umbilical cord. Blood and amniotic fluid were immediately centrifuged, and serum and supernatant were obtained, respectively. The sFas and sFasL were assayed in 10 and 17 patients, respectively, by an enzyme-linked immunosorbent assay kit from Bioscience Laboratory Research Center, Mochida Pharmaceutical Co., LTD., Tokyo, Japan [8]. Although the sFas assay fluctuation was not evaluated, the coefficient of variation for the intra-assay and inter-assay of sFasL ranges from 2.4–11.7% (mean 6.8%) and 8.3–15.7% (mean 11.0%), respectively.

Data are expressed mean±SD. Comparison between values of measurement sites was analyzed by one-way analysis of variance, followed by Scheffe's F-test for multicomparison. Correlation analysis was made by Pearson's correlation coefficient. $p<0.05$ was considered significant.

Results

The age and gestational duration in subjected parturients were 31.6±4.6 (range 22–40) yr and 38.4±0.7 (range 38–40) w. No parturients showed any clinical problems during and after anesthesia, and their neonates also presented sufficient Apgar scores at delivery and no clinical problems with feeding and other post-natal behavior. The neonatal weight was 3,142±351 g.

The results of sFas and sFasL in each measurement site are shown in Fig. 1. The sFas levels in maternal arterial serum, umbilical venous serum, umbilical arterial serum and amniotic fluid were 2.1±3.4, 1.3±3.1, 1.3±3.0 and 0.2±0.4 ng/mL, respectively. There was no statistically significant difference between these values ($p=0.4937$).

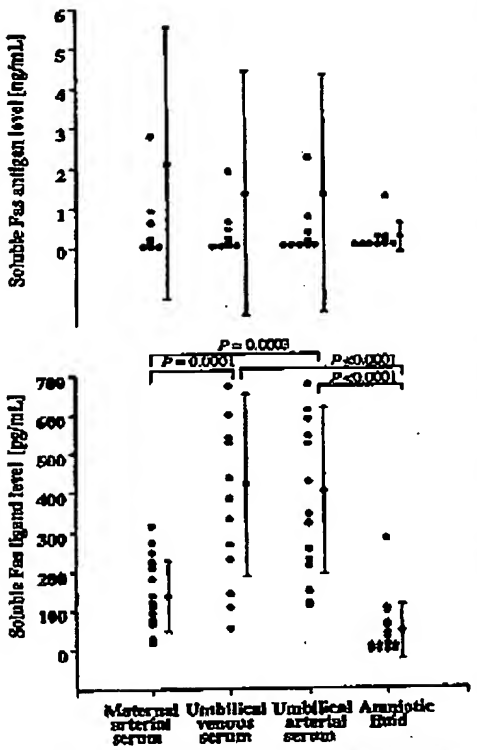

Fig. 1 Soluble Fas antigen (n=10) and soluble Fas ligand (n=17) levels. Data are individual values and mean±SD

The sFasL levels in maternal arterial serum, umbilical venous serum, umbilical arterial serum and amniotic fluid were 135.2±89.1, 419.6±232.8, 402.4±212.9 and 43.4±70.2 pg/mL, respectively. There was a significant difference between these values (p<0.0001). Umbilical venous and arterial sera levels were significantly higher than maternal arterial serum and amniotic fluid levels.

In each measurement site, there was no statistical correlation between sFas and sFasL levels. Within sFas levels, there were linear correlation between all measurement sites, in which maternal arterial serum vs umbilical venous serum level, maternal arterial serum vs umbilical arterial serum level, maternal arterial serum vs amniotic fluid level, umbilical venous serum vs umbilical arterial serum level, umbilical venous serum vs amniotic fluid level, and umbilical arterial serum vs amniotic fluid level indicated 0.0017/0.83, 0.0016/0.832, 0.008/0.763, <0.0001/0.999, <0.0001/0.952 and <0.0001/0.945 of p/r values, respectively. Within sFasL levels, there were linear correlation between maternal arterial serum and amniotic fluid level (p=0.0107, r=0.593), between umbilical venous serum and umbilical arterial serum level (p<0.0001, r=0.886), between umbilical venous serum and amniotic fluid level (p=0.0249, r=0.537), and between umbilical arterial serum and amniotic fluid level (p=0.0454, r=0.489), but there were no correlation between maternal arterial serum and umbilical venous or arterial serum levels.

Discussion

The Fas, a member of the tumor necrosis factor/nerve growth factor receptor family, is a type I membrane protein [14, 15], and is abundantly expressed in liver, heart, lung, kidney and ovary [24], and is up-regulated in hepatocytes transformed by human hepatitis C virus [23], in activated mature lymphocytes and in lymphocytes transformed by Epstein-Barr, human T-cell leukemia and human immunodeficiency viruses [5, 7, 10, 14, 20, 21]. The FasL, a member of the tumor necrosis factor family, is a type II membrane protein and is predominantly expressed in activated or cytotoxic T-lymphocytes, natural killer cells and neutrophils [1, 3, 8, 12, 14, 16, 20]. The FasL expressed on the surface of these cells then binds to Fas on the target cells and induces apoptosis [14]. Although the relationship between the membrane and soluble forms of Fas and FasL has not yet been elucidated, it has been suggested that sFas exerts a suppressive effect on Fas-FasL mediated apoptosis [4, 14]. On the other hand, sFasL, which is cleaved from FasL by matrix metalloproteinase [11], consists of the extracellular region of FasL which then binds to Fas on target cells to induce apoptosis [19]. The administration of recombinant human sFasL results in significantly more rapid induction of apoptosis in Fas-expressing T-lymphocytes and fibroblast cell lines in mice [17, 21]. However, recent reports [18, 22] have described that sFasL, in part, exerts a suppressive effect on Fas-FasL mediated apoptosis. Allgaier et al. [2] reported that apoptosis is diminished in umbilical cord blood neutrophils compared to adult cells. Taking these reports into consideration, the increase in sFas level may indicate diminution of apoptosis, whereas the increase in sFasL level may have both augmentation and diminution of apoptosis. We speculate that an acceleration of Fas-FasL mediated apoptosis results in the increased sFasL levels, which subsequently inhibits some apoptotic reactions, for instance neutrophils apoptosis, probably in order to preserve some immune homeostasis.

This study showed that neonatal serum sFasL level is significantly higher than maternal serum level, although neonatal and maternal serum sFas levels showed no difference. Normal serum levels of sFas and sFasL in adult seem to be less than 0.6 ng/mL and 200–300 pg/mL, respectively [9]. Based on these values, neonatal and maternal sFas levels were high, and maternal sFasL level was relatively low, whilst neonatal sFasL level was ap-

parently high. Furthermore, neonatal sFas levels correlated to maternal levels, but neonatal sFasL levels did not correlate to maternal levels. Drénou et al. [6] reported that FasL and Fas expressed in umbilical cord blood lymphocytes are present and absent or low, respectively. Since a non-activated lymphocyte expresses no FasL, this report may be consistent with the present result of high sFasL levels in cord blood. Furthermore, since the absent or low Fas in lymphocyte results in its less apoptosis, this report can also explain why cord blood neutrophils show diminished apoptosis [2], if this neutrophils expresses no Fas. From these results and implications, full term parturients may be undergoing a relatively diminished condition of Fas-FasL mediated apoptosis, whilst neonate or fetus may be undergoing an relatively accelerated condition of Fas-FasL mediated apoptosis, as a whole. This hypothesis may indicate immune privilege for mother, and for neonate or fetus vestige during ontology, placental degradation or changed circulation system after delivery. Further study is needed to elucidate such hypothesis.

In conclusion, neonatal serum sFasL level is significantly higher than maternal serum level, although neonatal and maternal serum sFas levels are similar. Neonatal sFasL level does not correlate to maternal sFasL level. Further study to elucidate the evidence of high serum sFasL levels in umbilical cord blood is needed.

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Zeichen/Ref./Réf.	Anmeldung Nr./Application No./Demande n°/Patent Nr./Patent No./Brevet n°.
BR74891/DC1/JT/	01959894.5-2405- PCT/US0141666

Anmelder/Applicant/Demandeur/Proprietaire/Patentinhaber/Proprietario/Titulaire
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PROCEEDING FURTHER WITH THE EUROPEAN PATENT APPLICATION PURSUANT TO ARTICLE 96(1) AND RULE 51(1) EPC

A supplementary European search report has been drawn up concerning the above European patent application (publication no. 1309615).

Since you have filed a request for examination prior to the transmission of the supplementary European search report, you are hereby invited to indicate within

TWO MONTHS

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